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# Neurite outgrowth promoting activity of marine algae from Japan against rat adrenal medulla pheochromocytoma cell line, PC12D

Yuto Kamei* & Atsuko Sagara
*Marine and Highland Bioscience Center, Saga University, 152-1 Shonan-cho, Karatsu, Saga, Japan*
*(* Author for correspondence; E-mail: kameiy@cc.saga-u.ac.jp; Fax: 0955 77 4486)*

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**Abstract**

We screened for biological activity which induces neurite outgrowth *in vitro* from 300 species of marine algae from along the Japan coast for possible use as a treatment for the lack of neurotrophic factor which is considered to be a cause of Alzheimer's disease. In this study, we evaluated the neurite outgrowth promoting activity in a rat adrenal medulla pheochromocytoma cell line, PC12D, using a low level of NGF (nerve growth factor). Although most of the samples had no activity, MeOH extract from a brown alga, *Sargassum macrocarpum* and PBS extract from a red alga, *Jania adharens*, exhibited neurite outgrowth promoting activity and induced neuron specific dendrites and axons from the surfaces of PC12D cells. The active substance present in *S. macrocarpum* seemed to be lipid and heat stable with molecular weight of around 500 to 1000. These results suggest that marine algae may constitute a good source for development of promising novel agents with neurotrophic activity in brain nerve systems for future use in treatment of Alzheimer's disease.

*Abbreviations:* NGF, nerve growth factor; FGF, fibroblast growth factor; EGF, epidermal growth factor; IGF, insulin-like growth factor; CVD, cerebrovascular disease; BDNF, brain derived neurotrophic factor; CNTF, cilialy neurotrophic factor; NT-3, neurotrophin-3; MeOH, methanol; PBS, phosphate buffered-saline.

## Introduction

Neurons need essential neurotrophic factors to differentiate and survive, and most of these factors are glycoproteins. Each neurotrophic factor is produced in glia cells or surrounding cells adjacent to the axons of neurons and retransported from the tip of the axons into neuron cell bodies, performing the roles of maintaining survival and promoting differentiation. The first discovered neurotrophic factor was NGF (nerve growth factor) which acts on sympathetic or sensory nerve systems at an early viviparous stage and also on basal forebrain cholinergic neurons (Thoenen and Brade, 1980). Since the discovery of NGF, investigations of neurotrophic factors have expanded and other neurotrophic factors such as BDNF (brain derived neurotrophic factor) (Brade et al., 1982), which acts on sensory neurons that do not respond to NGF, CNTF (cilialy neurotrophic factor) (Watters and Hendy, 1987) which acts on parasympathetic neurons and NT-3 (neurotrophin-3) (Hohn et al., 1990) to NT-4 and NT-5 belonging to these gene families have recently been discovered.

Furthermore, it is known that cell growth factors, FGF (fibroblast growth factor) (Morrison et al., 1986), EGF (epidermal growth factor) (Morrison et al., 1987) and IGF (insulin-like growth factor) (Aizerman and De Velli, 1987) maintain survival of cultured neurons and act in a manner similar to that of neurotrophic factors. In addition to these glycoproteins, thyroid hormone tyrosine (Hayashi and Patel, 1987), amino acid and a steroid ovarian follicle hormone estradiol (Arimatsu and Hatanaka, 1986) are also known as substances which show neurotrophic-like actions. Thus,

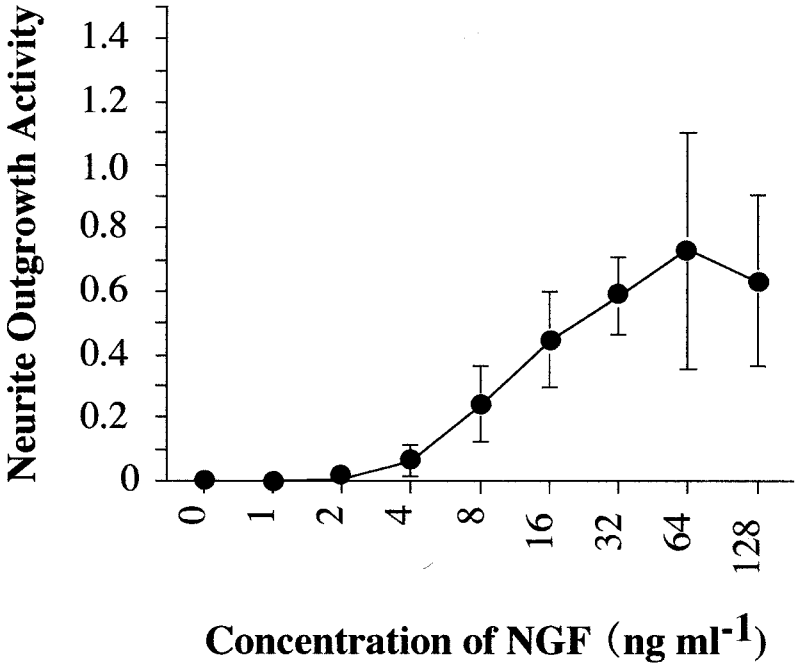


*Figure 1.* Dose-dependent neurite outgrowth activity of NGF to PC12D cells.

each neurotrophic factor is indispensable for the differentiation and maintenance of neurons. As described above, the lack of the neurotrophic factor in the brain suggests that the reduced synthesis of neurotrophic factors results in degeneration of neurons and further shrinkages of dendrites or whole cerebral cortex contractions, which are regarded as a cause of Alzheimer's disease. In particular the neurotrophic factor, NGF, was demonstrated to act on cholinergic neurons of Meynert basal ganglia (Whitehouse & Stube, 1982), and to maintain survival and promote differentiation. It is reported that when NGF is directly administered to the brain of Alzheimer's disease patients, the disease is temporally recovered (Olson et al., 1992). Thus, when NGF is lacking, it is expected that administration of neurite outgrowth promoting substances will inhibit the degeneration of neurons.

As the discovery of other neurite outgrowth promoting substances might possibly lead to a treatment for method of Alzheimer's disease, we began to screen for new neurite outgrowth promoting activity from seaweeds as a good potential natural source. Seaweeds have already been used as foods and a component of herbal medicines especially in oriental countries. Thus, they might constitute an exciting and extremely promising bioresource for new bioactive substances which have no side-effects.

In this study, we attempted *in vitro* screening for neurite outgrowth promoting activity in PC12D cells derived from rat adrenal medulla pheochromocytoma with extracts from a total of 300 species of marine algae collected along the Japan coast and further evaluated the dose-dependent activity of the active seaweeds to PC12D cells.

## Materials and methods

### *Algae extracts*

Algae samples were collected by skin-diving from 79 points along the Japan coastline (Harada et al., 1997) and washed 3 times with sterilized artificial seawater (ASW, Jamarin Laboratory) and once with phosphate buffered saline (PBS, pH 7.2). Twenty ml of PBS was added to 5 g of each algal sample for homogenization (8000 rpm, 5 min, room temperature) by Polytron (Kinematica Inc.) and the samples were centrifuged (1600 × g, 20 min, room temperature). The supernatants were collected and an additional 20 ml of MeOH was added to the pellets. The samples were homogenized as described above to prepare MeOH-extracts. Each PBS- and MeOH-extract was sterilized with a 0.2 $\mu$m-pore-sized membrane filter (Millipore, filter type JG).

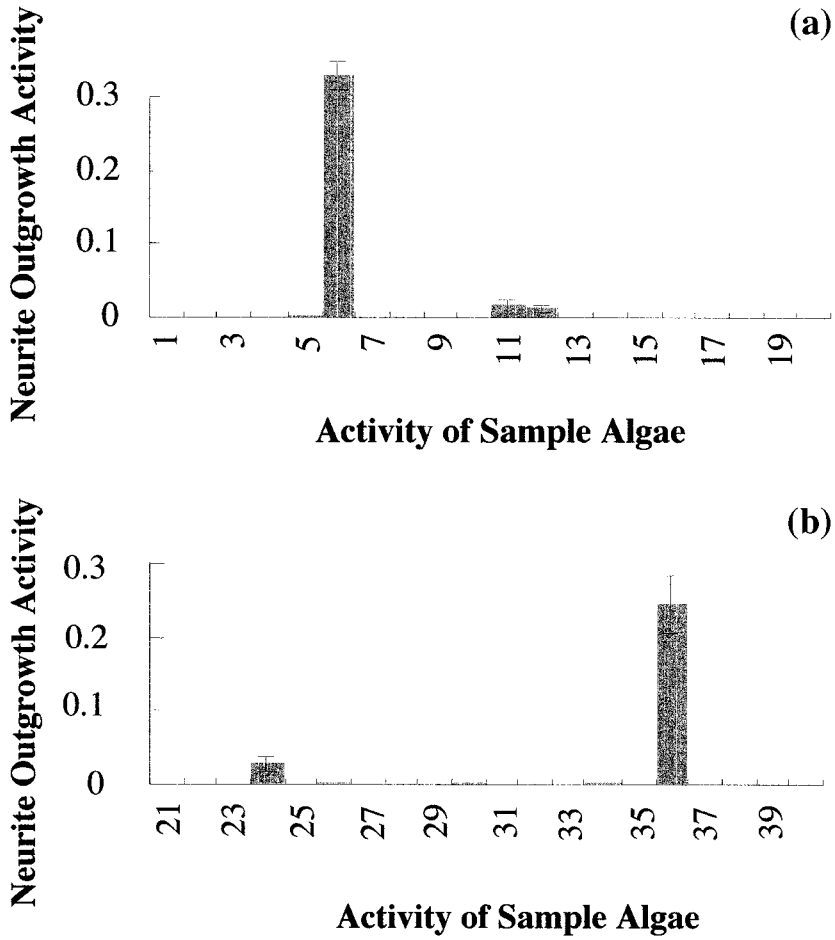


*Figure 2.* (a) Screening example for neurite outgrowth promoting activity of MeOH extract from marine algae in PC12D cells. Algal sample number: 1, *Sargassum horneri*; 2, *Dictyopteris membranacea*; 3, *Pachydictyon coriaceum*; 4, *Myagropsis myagroides*; 5, *Dictyopterisundulata*; 6, *Sargassum macrocarpum*; 7, *Colpomenia siuuosa*; 8, *Colpomenia bullosa*; 9, *Dictyota dichotoma*; 10, *Sargassum hunbergii*; 11, *Sargassum yendoi*; 12, *Sargassum yezoense*; 13, *Zonaria stipitata*; 14, *Agamum cribrosum*; 15, *Eisenia bicyclis*; 16, *Sphaerotrichia divaricata*; 17, *Isige okamurai*; 18, *Sargassum hemipyllum*; 19, *Padina minor*; 20, *Padina arbo rescens*. (b) Screening example for neurite outgrowth promoting activity of PBS extracts from marine algae in PCI2D cells. Algal sample number: 21, *Ecklomia cava*; 22, *Scytosiphon lomentaria*; 23, *Zonaria diesingiana*; 24, *Sargassum nipponicum*; 25, *Hizika jusiformis*; 26, *Sargassum alteronatopinnatum*; 27, *Sargassum confusum*; 28, *Dicryopteris prolifera*; 29, *Sargassum fulvellum*; 30, *Nemacystis decipiens*; 31, *Sargassum patens*; 32, *Tubinaria ornata*; 33, *Undaria pinnatifida*; 34, *Asparagopsis axiformis*; 35, *Polysiphonia morrowii*; 36, *Jania adharens*; 37, *Chondria crassicaulis*; 38, *Hypnea charoides*; 39, *Amphirosa dilatata*; 40, *Gelidiella acerosa*.

*Screening method*

We used PC12D cells derived from a rat adrenal medulla pheochromocytoma which were kindly provided by Dr. Mamoru Sano (Sano and Iwanaga, 1994) of the Institute for Developmental Research, Aichi Human Service Center. NGF (2.5S) was purchased from Sigma. Cells were maintained and cultured in E-RDF medium (Kyokuto) containing 10% fetal bovine serum (FBS) at 37 °C in a 5% $CO_2$ incubator. For determining the minimum active concentration of NGF, PC12D cells were treated with 0, 1, 2, 4, 8, 16, 32, 64 and 128 ng $ml^{-1}$ NGF and the neurite outgrowth activity was measured by the same method of screening section. The minimum concentration of NGF at which neurite outgrowth was observed was noted. PC12D cells ($5 \times 10^3$ 100 $\mu l^{-1}$ $well^{-1}$) were seeded and cultured at 37 °C for 24 h, after which the medium was replaced with fresh E-RDF medium containing 10% FBS and 2.5 ng $ml^{-1}$ NGF. At the same time, 5 $\mu$l of each algal extract was added and incubated at 37 °C. After 48 h, we counted cell numbers under a microscope (×200 magnification), and cells

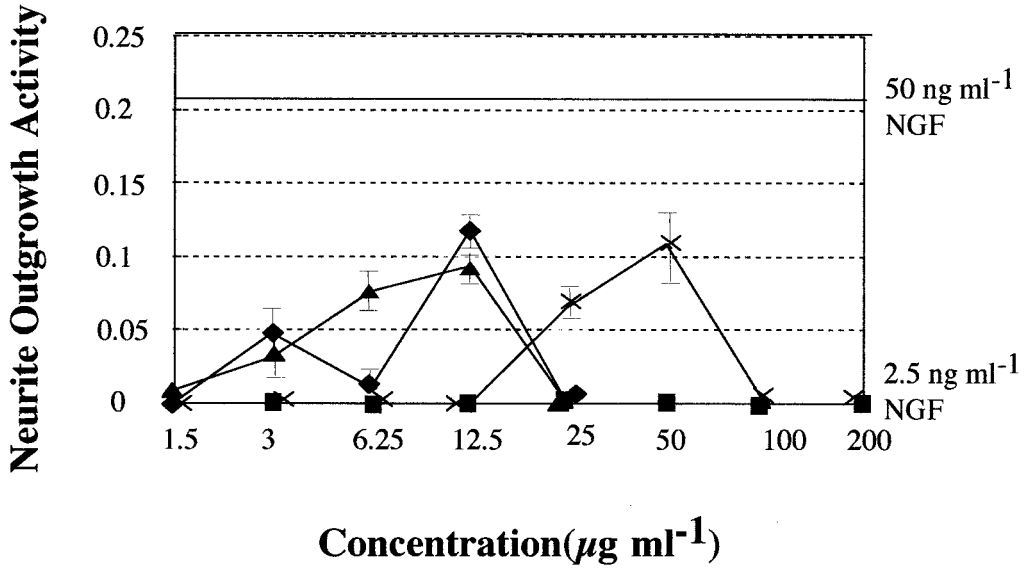


*Figure 3.* Neurite outgrowth promoting activity of organic solvent extracts from *Sargassum macrocarpum*. —♦—, MeOH extract; —▲—, chloroform extract; —■—, hexane extract after MeOH extraction; —×—, hexane extract.

bearing neurites which were twice as long as the whole cell body. The values were defined by the following formula for neurite outgrowth promoting activity. Two wells per one sample in a 96-well plate were examined and 4 points in each well were evaluated for neurite outgrowth promoting activity. The neurite outgrowth activity was estimated in the presence of the minimum active concentration of NGF (2.5 ng $ml^{-1}$).

Neurite outgrowth promoting activity =

cell numbers bearing neurites/cell numbers

*Organic solvent extracts from Sargassum macrocarpum*

After extraction from *S. macrocarpum* with PBS, MeOH, chloroform or hexane was directly added to the pellets and extraction was carried out as described above. Furthermore, after extraction with MeOH, an equal volume of hexane was further added to the pellet and extraction was carried out as described above to confirm the remaining activity in the MeOH-extracted pellet. Each solvent-extract was dried and redissolved in MeOH to give a concentration of 4 mg $ml^{-1}$. Each diluted extract was added to the cell culture to give a final concentration of 1.5 to 200 $\mu$g $ml^{-1}$ in the presence of 2.5 ng $ml^{-1}$ NGF.

*Characteristics of active substance in S. macrocarpum*

For investigating the resistance of active substance against heat, we prepared the active samples in medium maintained at 4 °C and those heated at 20, 30, 40, 50, 60, 70, 80, 90 and 100 °C for 10 min, and treated by autoclave one. Measurement of each activity was performed at 200 to 1.5 $\mu$g $ml^{-1}$. To know the rough molecular weight of active substance, we compared the result of gel filtration chromatography of active substance with that of already known substance such as vitamin $B_{12}$ (Katayama Chemical), and erythromycin (Wako Pure Chemical Industries). On Sephadex LH-20, substances were eluted with 90% MeOH at 3 ml $min^{-1}$.

## Results

*Minimum active concentration of NGF*

We initially investigated the minimum active concentration of NGF required to estimate *in vitro* neurite outgrowth promoting activity of marine algal extracts under the assumption of a lack of NGF in Alzheimer's disease. In confirmation of minimum neurite outgrowth activity of NGF in PC12D cells in the presence of 0 to 128 ng $ml^{-1}$ NGF, NGF showed the activity at concentrations of more than 2 ng $ml^{-1}$ (Figure 1).

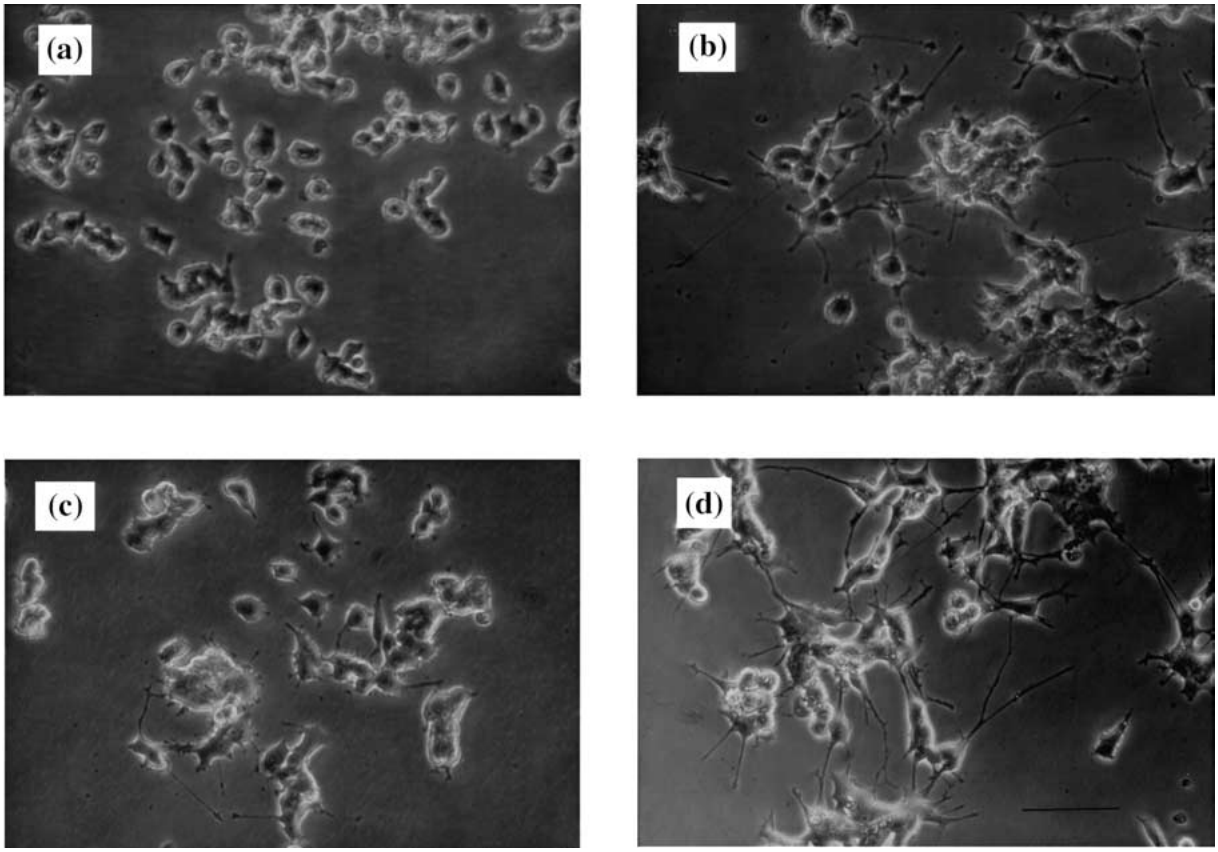


*Figure 4.* Photomicrographs of PC12D cells with neurites promoted by marine algal extract. (a) Untreated PC12D cells. (b) PC12D cells in the presence of 50 ng $ml^{-1}$ NGF. (c) PC12D cells in the presence of 2.5 ng $ml^{-1}$ NGF. (d) PC12D cells in the presence of 2.5 ng $ml^{-1}$ NGF plus 12.5 $\mu$g $ml^{-1}$ chloroform-extract from a brown alga, *Sargassum macrocarpum*. Bar = 100 $\mu$m.

Therefore, we set up a screening assay for neurite outgrowth promoting activity of the algal extracts to PC12D cells in the presence of 2.5 ng $ml^{-1}$ NGF.

*Screening for neurite outgrowth promoting activity*

Screening for neurite outgrowth promoting activity in PC12D cells from 300 species of marine algae indicated that only MeOH extract from a brown alga, *Sargassum macrocarpum*, and PBS extract from a red alga, *Jania adharens*, exhibited prominent neurite outgrowth promoting activity (Figures 2a and b). The activity of *S. macrocarpum* was 0.33 and that of *J. adharens* was 0.24, while the control activity of 2.5 ng $ml^{-1}$ NGF showed 0.03, indicating that these two algae extracts had neurite outgrowth promoting activities that were 10 and 8 times higher than the control, respectively.

On the whole, there were few MeOH extracts that had an influence on neurite prolonging activity or morphological changes of PC12D cells. On the other hand, a number of PBS extracts inhibited neurite outgrowth activity, rather showing cytotoxicity. As described above, cytotoxicity or neurite outgrowth inhibiting activity was shown even with 1/10-diluted PBS extracts.

*Neurite outgrowth promoting activity of organic solvent extract from S. macrocarpum*

We examined the dose responses of organic solvent extracts from a brown alga, *S. macrocarpum*, to neurite outgrowth activity in PC12D cells. The results are shown in Figure 3. Each algal extract promoted dose-dependent neurite extension of PC12D cells in the presence of the minimum active concentration of NGF (2.5 ng $ml^{-1}$). In particular, chloroform and MeOH extracts exhibited prominent neurite outgrowth promoting activity to PC12D cells at lower concentrations from 3 to 12.5 $\mu$g $ml^{-1}$, though cytotoxicity was ob-

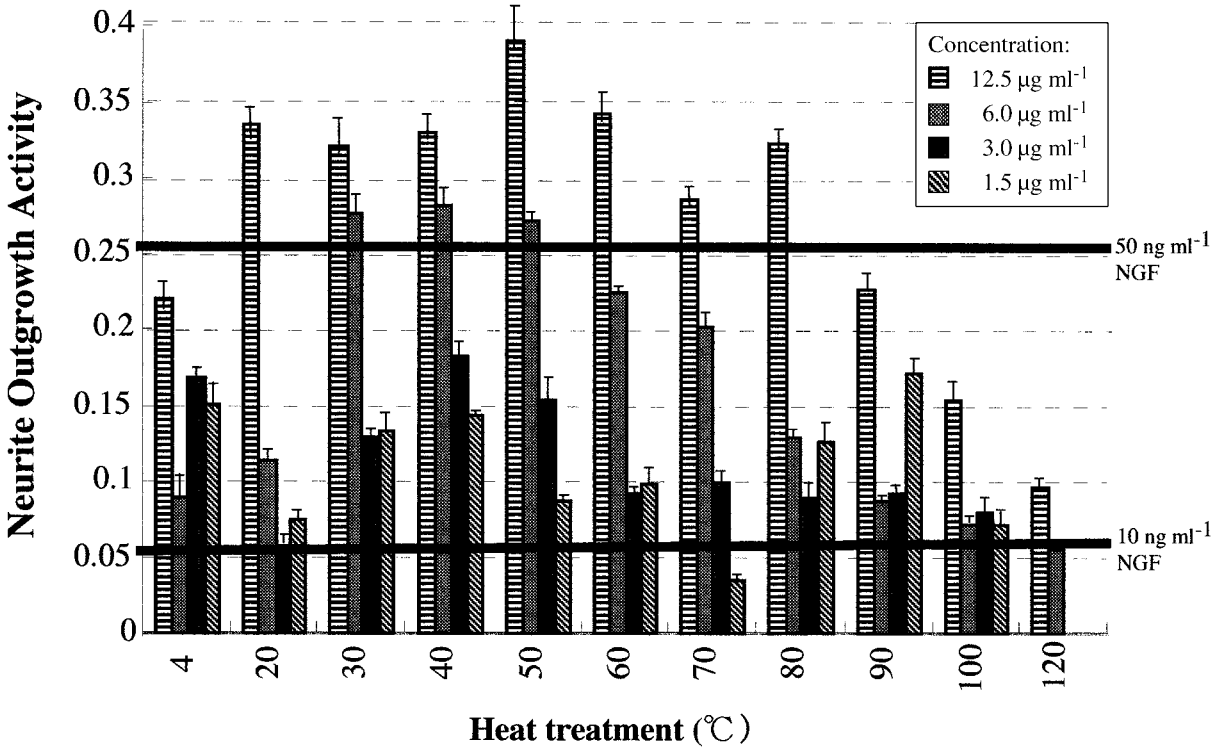


*Figure 5.* Heat stability test of neurite outgrowth promoting substance from *S. macrocarpum*. Chloroform extract was treated at 4 to 121 °C.

served at greater than 25 $\mu$g ml$^{-1}$. However, neurite outgrowth was not shown in the absence of NGF. Furthermore, hexane extract following MeOH extraction of *S. macrocarpum* showed no such activity, though one-step extract did show activity, indicating that the main active compounds might be soluble in MeOH, but not in hexane. Some of the cell morphologies are shown in Figure 4. These results suggested that this algal extract would require the existence of at least 2.5 ng ml$^{-1}$ NGF in the culture to exhibit neurite outgrowth promoting activity in PC12D cells.

*Heat stability and approximate molecular weight*

In the examination for heat resistance of chloroform extract from *S. macrocurpum*, which showed most dose-dependent neurite outgrowth activity in PC12D cells, extract treated at 4 °C until 80 °C showed activity but samples heated at 90 °C, 100 °C, and treated by autoclave showed lower activity (Figure 5). Either in this examination, the cytotoxicity was observed at the concentration over 25 $\mu$g ml$^{-1}$. As a result of gel filtration chromatography of active substance from *S. macrocurpum* using vitamin $B_{12}$ (MW 1355.38) and erythromycin (MW 733.94) as the molecular markers, the peak of active substance could be monitored by UV at 210 nm and appeared between that of vitamin $B_{12}$ and that of erythromycin (Figure 6), demonstrating the neurite outgrowth promoting activity. Therefore, it was roughly estimated that the molecular weight of active substance might be around 500–1000.

## Discussion

Since the aging of societies in developed countries might worsen in the 21st century, it will clearly be desirable to overcome some problems like Alzheimer's disease. Thus, in this study we attempted to screen for neurite outgrowth activity using a low level of NGF from marine algae which are expected to provide a source of many as yet unknown useful physiological substances to establish a treatment for Alzheimer's disease.

We found only two positive activities, one in MeOH extract from a brown alga, *Sargassum macrocarpum*, and the other in a PBS extract from a red alga, *Jania adherens*, among 600 samples composed of 300 species of marine algae, including 8 species

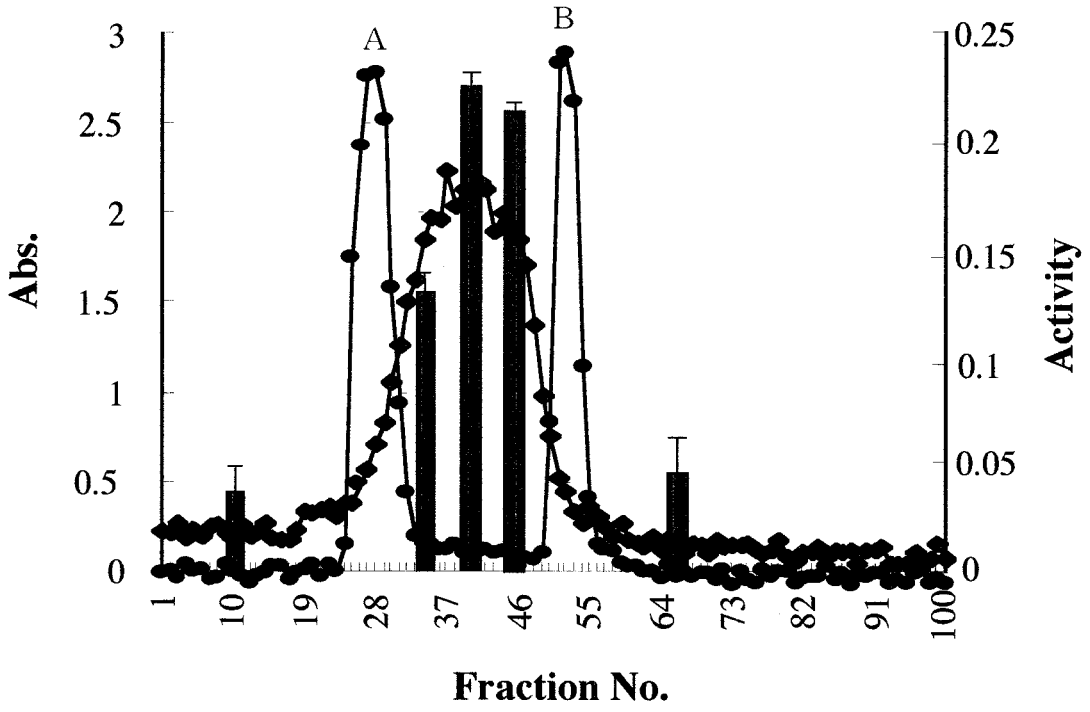


*Figure 6.* Gel filtration chromatography of neurite outgrowth promoting substance from *S. macrocarpum*. Neurite outgrowth promoting substance in the extract from *S. macrocarpum* was run on a Sephadex LH-20 column (25 mm × 765 mm). The molecular weight of active substance was estimated as around 500–1000 in comparison with Vitamin B12 (MW 1355.38) and Erythromycin (MW 733.94) as molecular size markers. —♦—, Neurite outgrowth promoting substance from *S. macrocurpum*. Abs. (210 nm). —●—, MW marker. A: Vitamin B12 (MW = 1355.38); B: Erythromycin [MW = 733.94) Abs. (230 nm). ■, Activity of neurite outgrowth was measured at 3 mg $ml^{-1}$ in the cell culture after fractions were concentrated to dryness and dissolved again in MeOH.

of sea plants, collected from the Japan coast line. On the whole, few marine algal MeOH extracts influenced cell morphological development, while many of the algal PBS extracts rather inhibited neurite outgrowth. The active compounds present in *S. macrocarpum* could be extracted by chloroform as well as MeOH, indicating a relatively lower polar substance. Moreover, both chloroform and MeOH extracts from this alga exhibited strongly dose-dependent activity to PC12D cells in the presence of the minimum concentration of NGF. Since such neurite outgrowth activity derived from marine algae has not been reported yet, this investigation provides valuable information on this activity from certain seaweeds.

At present it is reported that Alzheimer's disease patient shows temporary recovery when NGF is directly administered into the brain. However, the molecular weights of most neurotrophic proteins such as BDNF or CNTF are large and similar to NGF (molecular weight 130 000) (Algeletti and Bradshaw, 1971). These molecules cannot pass through the blood vessels across the blood-brain barrier. Therefore, the administration of NGF is conducted only by direct pipe to the brain, but this is not desirable in consideration of the patients' physical discomfort. Thus, the administration of neurite outgrowth promoting substances might be conducted by crossing the blood-brain barrier using low molecular materials with neurotrophic activity to access the brain easily. Thus far, with the exception of growth factor proteins, several neurotrophic diffusion substances, thyroid hormone, tyrosine and ovarian follicle hormone, as well as estradiol are known to stimulate neurite outgrowth of neuron cells. However, these are still few in comparison to neurotrophic factor proteins.

In our screening, the active substance from a brown alga, *Sargassum macrocarpum*, could be extracted with MeOH or chloroform, and had heat resistance potency. Moreover the molecular weight of active substance was roughly estimated to be around 500 to 1000 based on the result of gel filtration chromatography. These data suggested that the neurite outgrowth promoting active substance that was newly discovered in this screening might be more effective to penetrate the

blood-brain barrier in comparison with usual protein neurotrophic factors.

As a mechanism of the promoting activity, it is thought that organic solvent extracts from *S. macrocarpum* might promote the permeating potency of NGF into the membrane of PC12D cells or strengthen binding affinity of NGF to receptors on the surface of PC12D cells. We will clarify this after purification of the active substances from the algal extract.

## References


Aizerman Y and De Velli J (1987) Brain neurons develop in serum and glial free environment: Effect of transferrin, insulin, insulin-like growth factor-I and thyroid hormone on neuronal survival, growth and differentiation. Brain 406: 32−42.

Algeletti H and Bradshaw A (1971) Nerve growth factor from mouse submaxilly gland: Amino acid sequence. Proc Natl Acad Sci USA 68: 2417−2420.

Arimatsu Y and H Hatanaka H (1986) Estrogen treatment enhances survival of cultured fetal rat amygdala neurons in a defined medium. Dev Brain Res 26: 151−159.

Brade A, Edgar D and Thoenen H (1982) Purification of a new neurotrophic factor from mammalian brain. EMBO J 1: 549−553.

Harada H, Noro T and Kamei Y (1997) Selective antitumor activity in vitro from marine algae from Japan coasts. Biol Pharm Bull 20: 541−546.

Hayashi M and Patel J (1987) An interaction between thyroid hormone and nerve growth factor in the regulation of choline acetyltransferase activity in neuronal cultures, derived from the septal-diagonal band region of the embryonic rat brain. Dev Brain Res 36: 109−120.

Hohn A, Leibrock K and Brade A (1990) Identification and characterization of a novel member of the nerve growth factor/brain derived neurotrophic factor family. Nature 344: 339−341.

Morrison S, Sharma A, Vellis D and Bradshaw A (1986) Basic fibroblast growth factor supports the survival of cerebral neurons in primary culture. Proc Natl Acad Sci USA 83: 7537−7541.

Morrison S, Kornblum I, Leslie M and Bradshaw A (1987) Trophic stimulation of cultured neuron from neonatal rat brain by epidermal growth factor. Science 238: 72−75.

Olson L, Nordberg A, Holst HV, Backman L, Ebendal T, Alafuzoff I, Amberla K, Hartvig P, Herlitz A, Lilja A, Lundqvist H, Langstrom B, Meyerson B, Persson A, Viitanen M, Winblad B and Seiger A (1992) Nerve growth factor affects 11C-nicotine binding, blood flow, EEG, and verbal episodic memory in an Alzheimer patient (Case Report). J Neural Transm 4: 79−95.

Sano M and Iwanaga M (1994) Local sprouting of neuritis from cultured PC12D cells in response to a concentration gradient of nerve growth factor. Brain Res 656: 210−214.

Thoenen H and Brade A (1980) Physiology of nerve growth factor. Physiol Rev 60: 1284−1335.

Watters D and Hendy A (1987) Purification of a ciliary neurotrophic factor from bovine heart. J Neurochem 49: 705−713.

Whitehouse JLP and Stuble G (1982) Alzheimer's disease and senile dementia: Loss of neurons in the basal forebrain. Science 215: 1237−1239.